

Inhibition of *Neisseria gonorrhoeae* by normal human saliva

A. MELLERSH, A. CLARK, AND S. HAFIZ

From the Department of Medical Microbiology, University of Sheffield Medical School, Sheffield

SUMMARY Saliva was found to be a powerful and specific inhibitor of *Neisseria gonorrhoeae*. Although 28 other species of bacteria were tested, including *Neisseria meningitidis*, *Neisseria pharyngis* var *flava*, *Neisseria lactamica*, and *Neisseria catarrhalis*, we failed to find any others sensitive to saliva under similar conditions. The physical properties of the inhibitory substance indicated that it might be salivary α -amylase. To test this hypothesis α -amylase was extracted from saliva and was shown to have a high antigonococcal activity. Hog pancreas α -amylase also showed strong antigonococcal activity, thus the observations indicate that for the strains we tested α -amylase is inhibitory to gonococci. This observation indicates that either the gonococcal outer cell wall contains some unique lipopolysaccharides or that the gonococcus is unusually dependent on the integrity of these moieties. Whichever speculation proves to be true it indicates a need for a careful study of the gonococcal cell wall.

Introduction

Gonococcal infection of the pharynx is becoming increasingly common. It is usually asymptomatic and may be resistant to normal treatment regimens, since results of tonsillar swabs remain positive after those from other sites have become negative (Wiesner, 1975). Many clinicians believe that undiagnosed or resistant pharyngeal carriage presents an important residual pool of infection. In Denmark, Bro-Jørgensen and Jensen (1973) showed that in 10% of women with cervical gonorrhoea the organism could also be isolated from tonsillar swabs.

It has been known for many years that the gonococcus can infect the pharynx. However, until now, the effect of saliva on the organism has not been examined. Gordon (1916) tested the effect of sterile saliva on the meningococcus and found no inhibition of growth. Bibby *et al.* (1938) reported the action of saliva on 169 strains of bacteria but unfortunately did not include the gonococcus among these.

This study was first undertaken to examine the effects of saliva on *Neisseria gonorrhoeae*. The

inhibitory effect, which was consistently observed, was then further studied in order to characterise it and determine its nature.

Material and methods

SALIVA SPECIMENS

A total of 65 specimens of whole mouth unstimulated saliva was studied. Of these, 35 specimens were obtained from patients attending the Sheffield special treatment clinic and 30 were from healthy controls; the age range was from 20 to 60 years. Pure parotid saliva was obtained from one individual by means of a Curby cup placed over the parotid duct. A pool of saliva for more detailed analysis was collected from one individual. All saliva samples were sterilised by Seitz filtration.

BACTERIA STUDIED

Neisseria gonorrhoeae

A total of 13 strains of *N. gonorrhoeae* was studied; five were international strains F62, K2, and WHO strains III, V, and VII; three were β -lactamase-producing strains, HK1, L6, L7 (kindly provided by Dr A. Percival) (HK1 was the strain selected to test all saliva specimens and to study the effect of saliva in detail); and the other five were fresh isolates from the throats of women attending the Sheffield special treatment clinic, designated R34, R37, R38, R40,

Address for reprints: A. Mellersh, Department of Medical Microbiology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX

Received for publication 15 August 1978

and R44. All strains were preserved by suspension in 1% proteose-peptone broth with 8% glycerol and were stored in the vapour phase of liquid nitrogen.

Other bacteria

The other species of *Neisseria* studied are as follows:

Gram-positive cocci

Staphylococcus Oxford
Staphylococcus 80/81
Micrococcus luteus NCIB 9278
 Streptococcus group A
 Streptococcus group D
Streptococcus viridans
Diplococcus pneumoniae
Sarcinia

Gram-negative cocci

Neisseria meningitidis
Neisseria catarrhalis
Neisseria pharyngis var *flavus* NCTC 4590
Neisseria lactamica

Gram-positive bacilli

Corynebacterium xerosis
Listeria monocytogenes
Lactobacillus plantarum NCIB 6376
Bacillus subtilis
Bacillus cereus
Clostridium welchii
Clostridium septicum

Gram-negative bacilli

Escherichia coli
Shigella sonnei
Salmonella typhimurium
Proteus mirabilis
Klebsiella pneumoniae
Pseudomonas aeruginosa
Haemophilus influenzae
Bacteroides melaninogenicus
Bacteroides fragilis

No inhibition was demonstrated.

CULTURE AND INHIBITION OF GROWTH BY SALIVA

The solid medium used for *N. gonorrhoeae* was Difco GC base plus 2% defined supplement (L-glutamine 1.0 g, dextrose 40.0 g, ferric nitrate 0.05 g, and cocarboxylase 0.02 mg per 100 ml distilled water). When the other bacteria studied failed to grow on the GC medium other suitable

media were used. Thus, *Haemophilus influenzae* was tested on chocolate agar and *Bacteroides* species anaerobically on modified BM agar plus 5% lysed blood.

Inhibition of growth on solid medium was tested as follows. A well-plate technique was used with 4 mm wells cut in the solid medium in standard Petri dishes. Each well was filled with saliva, which was allowed to diffuse into the medium for four hours at 4°C, after which time any remaining saliva was removed with a Pasteur pipette. The Petri plates were then flooded with an inoculum of the test organism suspended in saline and containing approximately 10^7 organisms per ml and any excess was then removed. After incubation for 18 hours at 37°C in 10% CO₂ with raised humidity the purity of growth was checked by Gram stain and the degree of growth inhibition was recorded.

The liquid medium used for the growth of *N. gonorrhoeae* was ANM (Hafiz and McEntegart, 1976) plus 2% defined supplement. In order to measure the inhibitory effect of saliva an initial 1/10 dilution of sterile saliva in ANM medium was made and doubling dilutions prepared in the same medium to a final dilution of 1/40 960. Each of these dilutions was inoculated with approximately 10^8 organisms in 0.1 ml of ANM.

Clostridia species were tested on reinforced clostridial medium (Oxoid Ltd, 1976) plus 10% saliva and incubated anaerobically.

All cultures were incubated for 18 hours at 37°C and purity of growth confirmed by culture and microscopy.

SURVIVAL OF *N. GONORRHOEAE* IN SALIVA

A heavy suspension of gonococci containing approximately 5×10^7 organisms/ml was prepared in ANM and divided into two 2-ml aliquots; to one, an equal volume of fresh sterile saliva was added and, to the other, an equal volume of saliva that had been heated to 90°C for five minutes. Both specimens were kept at 37°C and aliquots were taken from each at intervals for culture in ANM and for examination by phase contrast microscopy.

FURTHER EXAMINATION OF SALIVA IN RELATION TO *N. GONORRHOEAE* (HK1)

The effect of saliva on the gonococcus was assayed before and after the following procedures.

Effect of heat

Aliquots of whole saliva were heated for five minutes at temperatures from 40°C to 100°C at 5°C intervals. The aliquots were also assayed for α -amylase activity (see below).

Ultra-violet light

Fresh sterile saliva in an open Petri dish was exposed to ultra-violet light for five minutes.

Dialysis

Saliva was dialysed against 100 volumes of sterile saline overnight at 4°C.

Ammonium sulphate precipitation

Saturated ammonium sulphate was added slowly to aliquots of saliva so that the final concentrations were 30, 40, 55, and 70% saturation. Precipitates were centrifuged and resuspended in sterile saline. The precipitates and supernatants were dialysed against two changes of saline to remove ammonium sulphate before they were tested for antigonococcal activity.

PURIFICATION OF SALIVARY α -AMYLASE

This was performed according to the method of Meyer *et al.* (1947).

α -AMYLASE-ACTIVITY TEST

A starch solution of 5 mg/ml was prepared with 1% calcium lactate to replace any calcium ions that might have been lost during saliva purification. One millilitre of the saliva fraction was added to 5 ml of the substrate mixture and incubated at 56°C for 30 minutes. A saline control was incubated in parallel. The α -amylase activity was estimated visually after adding one drop of 1% iodine to test and control tubes.

Hog pancreas α -amylase and the purified salivary amylase were tested for antigonococcal activity by the well-plate technique.

FRACTIONATION OF ACTIVE ANTIGONOCOCCAL COMPONENT OF SALIVA

Whole mouth saliva was concentrated fifteenfold and 4 ml applied to a Sephadex G-75 column (Pharmacia, Uppsala, Sweden), 40 cm \times 2.0 cm, equilibrated with phosphate-buffered saline (0.15 mol/l, pH 7.2). The saliva was eluted with the same buffer and the eluate monitored for absorption at 280 nm with a LKB Uvicord II. Fractions were assayed by the techniques described above. Subsequently, hog pancreas α -amylase and purified salivary α -amylase were fractionated with the same apparatus.

Results

On solid media growth of all 13 strains of gonococci was inhibited by whole mouth saliva in a zone 16–20 mm in diameter round the well. Pure parotid saliva produced slightly greater inhibition. Different strains of gonococci showed variation in their

ability to grow in the presence of different saliva concentrations in liquid media (Table) but no strains grew in media with a saliva concentration of 1/320 or greater. All strains isolated from the throats of patients were strongly inhibited by saliva. No preponderance of any particular Kellogg type was noticed at the edge of the zone of inhibition nor was there any apparent correlation between Kellogg types and inhibition of growth by saliva.

All other bacteria tested grew freely in the presence of saliva.

Saliva from all 65 individuals tested strongly inhibited the HK1 strain of *N. gonorrhoeae*.

In survival tests gonococci were viable up to six hours after incubation with normal saliva and for up to 12 hours after incubation with heated saliva. No bacterial lysis was observed by phase contrast microscopy.

The inhibitory factor in saliva was destroyed by heating at 75°C for five minutes but not at 70°C for the same length of time; loss of α -amylase activity occurred at the same temperature. Exposure to ultra-violet light for five minutes did not alter activity. The active factor was not dialysable but was precipitated when ammonium sulphate was added to a concentration of 40% saturation. From these observations it seemed possible that the active factor might be salivary amylase. Chromatography of whole saliva, hog pancreas α -amylase, and purified salivary α -amylase is illustrated in the Figure. There were two peaks of antigonococcal activity in whole saliva and one in the hog pancreas α -amylase and the purified salivary α -amylase. The whole saliva second peak of antigonococcal activity coincided with that of the salivary α -amylase while that of the hog pancreas α -amylase was of slightly higher molecular weight. All peaks of antigonococcal activity had strong α -amylase activity.

Purified salivary α -amylase and hog pancreas α -amylase both showed strong antigonococcal activity.

Discussion

From the observations we have reported we believe that salivary α -amylase is a powerful and selective inhibitor of *N. gonorrhoeae*.

Table Growth of gonococcus strains in liquid media containing different concentrations of saliva

Saliva concentration	Strains growing
$\geq 1/320$	—
$\leq 1/640$	K2, L6, L7, WHO VII, R38
$\leq 1/1280$	WHO III, WHO V, R34, R37, R40, R44
$\leq 1/12560$	F62, HK1

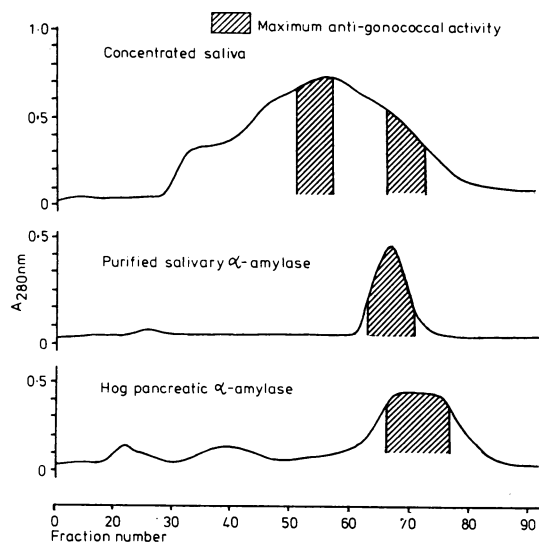


Figure 1. Tracings of whole-mouth saliva, hog pancreas α -amylase, and purified human salivary α -amylase gel-chromatographed on Sephadex G-75

The outer envelope of the gonococcal cell is generally believed to consist of three components, pili or fimbriae, protein subunits, and lipopolysaccharide units. Stead *et al.* (1975) compared the constituents of the cell wall lipopolysaccharides of commensal *Neisseria* species with those of gonococci and concluded that they were essentially similar.

If we assume that the α -amylase (approximately 50 000 Daltons) is acting on the outer cell wall and not passing into the gonococcus, a possible target for its action is the polysaccharide component of the lipopolysaccharide moieties. This may indicate

that either the structure of the gonococcal lipopolysaccharide is different from that of other *Neisseria* species (although the actual constituents may be the same) or the integrity of these moieties is especially important to the gonococcus. Possibly both mechanisms could account for the peculiar susceptibility of the gonococcus to α -amylase.

The authors are grateful to Professor M. G. McEntegart for his encouragement and help and to the staff of the Department of Medical Microbiology, University of Sheffield, for their assistance in this work. We are grateful to Dr A. Percival, Department of Medical Microbiology, University of Liverpool, for supplying some of the strains of gonococci and to the staff of the Special Treatment Clinic, Sheffield Royal Infirmary, for their co-operation and assistance.

A. Mellersh was supported by Difco on a student vacation fellowship.

References

- Bibby, B. G., Hine, M. K., and Clough, O. W. (1938). The antibacterial action of human saliva. *Journal of the American Dental Association*, **25**, 1290–1302.
- Bro-Jørgensen, A., and Jensen, T. (1973). Gonococcal pharyngeal infections: Report of 110 cases. *British Journal of Venereal Diseases*, **49**, 491–499.
- Gordon, M. H. (1916). The inhibitory action of saliva on growth of the meningococcus. *British Medical Journal*, **1**, 849–851.
- Hafiz, S., and McEntegart, M. G. (1976). Prolonged survival of *Neisseria gonorrhoeae* in a new liquid media. *British Journal of Venereal Diseases*, **52**, 381–383.
- Meyer, K. H., Fischer, E. H., Bernfeld, P., and Staub, A. (1947). Purification et cristallisation de l' α -amylase de salive. *Experientia*, **3**, 455.
- Oxoid Ltd (1976). *The Oxoid Manual of Culture Media: Ingredients and other Laboratory Services*, third edition.
- Stead, A., Main, J. S., Ward, M. E., and Watt, P. J. (1975). Studies on lipopolysaccharides isolated from strains of *Neisseria gonorrhoeae*. *Journal of General Microbiology*, **88**, 123–131.
- Wiesner, P. J. (1975). Gonococcal pharyngeal infection. *Clinical Obstetrics and Gynecology*, **18**, 121–129.